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(54) L-methionone-producing Microorganisms

(57) L-methionine-producing microorganisms are constructed by transforming, into a recipient strain of the genus *Escherichia,* a hybrid plasmid having had inserted therein a DNA fragment possessing genetic information relating to L-methionine production and derived from a donor strain of the genus *Escherichia,* which donor strain is resistant to an ethionine.

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The information required by Rule 17(1)(a)(iii) of the Patents Rules 1978 was not contained in the application as filed, but was supplied later in accordance with Rule 17(2).

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SPECIFICATION

L-Methionine-Producing Microorganisms

This invention relates to L-methionine-producing microorganisms constructed by a gene recombination technique.

Most wild microorganism strains do not produce L-methionine. In order to render a wild strain capable of producing L-methionine from carbohydrates, it is necessary to induce artificial mutants from the wild strain. There are many known methionine-producing artifical mutants. Typical known methionine-producing mutants are an ethionine-resistant mutant of *Escherichia coli* [J. Bacteriol., *76*, 326 (1958)]; an ethionine-resistant mutant of *Candida utilis* [Folia microbiol., *9*, 374(1964)]; ethionine-resistant, α-methylmethionine-resistant and norleucine-resistant mutants of *Salmonella typherium* [Genetics, *58*, 473 (1968)]; a norcleucine-resistant mutant of *Escherichicia coli* [Compt. Rend., *248*, 3490 (1959)]; and α-methylmethionine-resistant mutant of *Corynebacterium glutamicum* [U.S. Patent No. 3,729,381]; and an ethionine-resistant mutant of *Brevibacterium flavum* [Publication for Opposition of Japanese Patent Application No. 6753/1976].

The most efficient methionine-producer, as far as we know, is *Corynebacterium glutamicum* ATCC 21608 which produces 3.4 mg/ml of L-methionine from 10 g/dl of glucose. It has, however, become difficult to increase the yields of L-methionine using artificial mutation techniques.

According to the present invention, there is provided an L-methionine-producing microorganism obtained by incorporating, into a recipient strain of the genus *Escherichia*, a hybrid plasmid having had inserted therein a DNA fragment possessing genetic information relating to L-methionine production, which fragment is derived from a donor strain of the genus *Escherichia* resistant to ethionine.

The present invention also provides a process for producing L-methionine, which comprises culturing an L-methionine-producing microorganism of the invention.

The DNA-donor strain used to construct the L-methionine producer of this invention is a mutant of the genus *Escherichia* resistant to ethionine and possessing the genetic information relating to L-methionine production. Strains having a higher productivity of L-methionine are preferably used as the DNA-donor. The mutant resistant to ethionine used as the DNA-donor can be obtained by conventional mutation techniques.

Chromosomal DNA is extracted from the DNA donor in a known manner and treated with a restriction endonuclease by a known method [see, for example, Biochem. Biophys. Acta *383*, 457 (1975)]. The plasmid of phage DNA used as the vector in the synthesis procedure is also treated with a restriction endonuclease in an analogous manner. Various restriction endonucleases can be used, if the digestion of the chromosomal DNA is effected partially. Thereafter, the digested chromosomal DNA and the vector DNA are subjected to a ligation reaction. Recombination of the DNA to prepare the recombinant plasmid can be carried out by introducing, by the use of terminal transferase, deoxyadenylic acid and deoxythymidylic acid, or doxygualylic acid and deoxycytidylic acid, into the chromosomal DNA fragment and cleaved vector DNA, and by subjecting the modified chromosomal DNA fragment and the cleaved vector DNA to an annealing reaction.

As a suitable vector DNA, a conventional vector can be employed, such as Col E1, pSC 101, pBR 322, pACYC 177, pCR 1, R6K or λ-phage, or their derivatives.

The hybrid DNA thus obtained can be incorporated into a microorganism of the genus *Escherichia* by conventional transformation techniques [see, for example, J. Bacteriol., *119*, 1072 (1974)]. The desired transformant is screened by using a medium on which only a clone having one or both of the characteristics of L-methionine productivity originating from the chromosomal DNA fragment and from the vector DNA, can grow.

As the recipient microorganism for the hybrid DNA, an L-methionine-auxotroph is usually used, since it is conventional to distinguish the methionine-producing transformant from the recipient. Desirably, a mutant already having a higher productivity of L-methionine is used as the recipient, to obtain better results.

The methods of culturing the L-methionine-producing strains thus obtained are conventional, and are similar to the methods for the cultivation of known L-methionine-producing microorganisms. Thus, the culture medium employed may be a conventional one containing carbon sources, nitrogen sources, inorganic ions and, when required, minor organic nutrients such as vitamins or amino acids. Examples of suitable carbon sources include glucose, sucrose, lactose, starch hydrolysate and molasses. Gaseous ammonia, aqueous ammonia, ammonium salts and other nitrogen containing materials can be used as the nitrogen source.

The cultivation of the recombinant microorganisms is usually conducted under aerobic conditions, the pH and the temperature of the medium being adjusted to a suitable level, and may be continued until the formation of L-methionine ceases. The L-methionine in the culture medium can be recovered by conventional procedures.

By the method of the present invention, L-methionine can be produced in higher yields than has been achieved in previously known methods in which artificial mutants of *Escherichia* are used.

The invention will now be illustrated by the following Example.

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Example

(1) Preparation of Chromosomal DNA Possessing Genetic Information Relating to L-methionine Production

Escherichia col EG-20 (NRRL B-12392), a mutant resistant to S-(2-aminoethyl)-cysteine (AEC) and ethionine and derived from K-12 (ATCC 10798) by exposing K-12 cells to 250 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine in a citric acid buffer of pH 5.5 at 0°C for 60 minutes and separating the colony which appeared on the agar medium, was cultured at 37°C for 3 hours with shaking in 1 litre of L-medium containing 1 g/dl of peptone, 0.5 g/dl of yeast extract, 0.1 g/dl of glucose and 0.5 g/dl of NaCl (pH adjusted to 7.2), and bacterial cells in the exponential growth phase were harvested. Chromosomal DNA was extracted by a conventional phenol-method, whereby 3.4 mg of purified DNA were obtained.

(2) Preparation of Vector DNA

As the vector, DNA of pBR 322 was prepared as follows. A strain of *Escherichia coli* K-12 harbouring the plasmid pBR 322 was incubated at 37°C in 1 litre of a medium containing 2 g of glucose, 1 g of NH_4Cl, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl, 0.1 g of $MgSO_4 . 7H_2O$, 0.015 g of $CaCl_2 . 2H_2O$, 20 g of "casamino acid" (casein hydrolystate), 0.05 g of thymine, 0.05 g of L-tryptophan and 100 μg of thiamine . HCl, each per litre (pH adjusted to 7.2). After the strain had been incubated until the late log phase, 170 μg/ml of chloramphenicol was added to the culture medium. By this process, the plasmid DNA was amplified and accumulated abundantly in the bacterial cells.

After 16 hours of incubation, the cells were harvested and then lysed by treatment with lysozime and sodium dodecylsulphate. The lysate was centrifuged at 30,000 xg for 1 hour to obtain a supernatant. After concentrating the supernatant, 480 μg of the plasmid pBR 322 DNA was obtained by fractionation using cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

(3) Insertion of the Chromosomal DNA Fragment Into the Vector

Portions, each of 10 μg, of the chromosomal DNA were treated with the restriction endonuclease Hind III at 37°C for 5, 10, 20, 30 and 60 minutes, respectively, to cleave the DNA chains, and each portion was then heated at 65°C for 5 minutes. Portions, each of 10 μg, of the vector DNA were also each treated with the restriction endonuclease mentioned above at 37°C for 1 hour to cleave the DNA completely, and then heated at 65°C for 5 minutes.

The digested chromosomal DNA solution and cleaved vector DNA solution were mixed and subjected to a ligation reaction for DNA fragments by the use of T_4 phage DNA-ligase in the presence of ATP and dithiothreitol at 10°C for 24 hours. The reaction mixture was then heated at 65°C for 5 minutes, and a two-fold volume of ethanol was added to it. The precipitated recombinant DNA was recovered.

(4) Genetic Transformation With the Hybrid Plasmid Harbouring Genetic Information Relating to L-methionine Poduction

Recipient strains were induced from *Escherichia coli* K-12 by exposing to 250 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine in a citric acid buffer of pH 5.5 at 0°C for 60 minutes, the recipient strains induced being No. 2 (NRRL B-12393) (resistant to S-(2-aminoethyl)-cysteine), No. 30(NR) (NRRL B-12395) (resistant to norleucine), No. 77 (NRRL B-12397) (resistant to 2-thiazelealanine), and No. 283 (NRRL B-12398) (resistant to p-fluorophenylalanine).

Each of the recipient strains was inoculated in 10 ml of L-medium, and cultured at 37°C with shaking. Cells in exponential growth phase were harvested, and suspended in a 0.1M $MgCl_2$ solution and then in a 0.1M $CaCl_2$ solution in an ice bath, whereby "competent" cells having the ability of DNA uptake were prepared.

Into the competent cell suspension, the DNA obtained in step (3), which contains the hybrid plasmid DNA, was added. The suspension was kept in an ice-bath for 30 minutes, then heated at 42°C for 2 minutes, and again allowed to stand in an ice-bath for 30 minutes. The cells, thus having the hybrid plasmid DNA incorporated into them, were inoculated into L-medium and the medium was shaken at 37°C for 2 hours, whereby the transformation reaction was complete. The cells were harvested, washed, and resuspended. A small portion of the cell suspension was spread on an agar plate containing 2 g of glucose, 1 g of $(NH_4)_2SO_4$, 7 g of K_2HPO_4, 2 g of KH_2PO_4, 0.1 g of $MgSO_4 . 7H_2O$, 0.5 g of sodium citrate . $2H_2O$, 0.5 g of S-(2-aminoethyl)-cysteine, 20 μg/ml of ampicillin and 20 g of agar, each per litre except for the ampicillin, (pH adjusted to 7.2). The plate was incubated at 37°C for 3 days. Colonies appearing on the plate were picked up, and strains resistant to ampicillin, S-(2-aminoethyl)-cystein and ethionine were obtained.

Thus, L-methionine-producing hybrid clones AJ 11539 (FERM-P 5479, NRRL B-12399), AJ 11540 (FERM-P 5480, NRRL B-12400), AJ 11541 (FERM-P 5481, NRRL B-12401) and AJ 11542 (FERM-P 5482, NRRL B-12402) were obtained from the recipient strains No. 2, No. 30 (NR), No. 77 and No. 283 respectively.

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(5) Production of L-methionine by the L-methionine-producing Strains

The table below shows the experimental results of the fermentative production of L-methionine using the L-methionine-producing strains, the DNA-donor strain EG-20 and the recipient strains No. 2, No. 30 (NR), No. 77 and No.283.

The fermentation medium contained 5 g/dl of glucose, 2.5 g/dl of ammonium sulphate, 0.2 g of KH_2PO_4, 0.1 g/dl of $MgSO_4 . 7H_2O$, 0.05 g/dl of yeast extract, 100 μg/dl of thiamine . HCl, 1 mg/dl of $FeSO_4 . 7H_2O$, 1 mg/dl of $MnSO_4 . 4H_2O$ and 2.5 g/dl of $CaCO_3$ (separately sterilized), and its pH was adjusted to 7.0. Batches, each of 20 ml, of the fermentation medium were placed in 500 ml flasks, inoculated with one loopful inoculum of the test microorganism, and cultivation was carried out at 31°C to 72 hours. The amount of methionine in the supernatant of the fermentation broth was determined by microbiological assay.

Table

Microorganism tested	*L-methionine produced (mg/dl)*
EG-20	3
AJ 11539	10
AJ 11540	25
AJ 11541	18
AJ 11542	30
No. 2	2
No. 30 (NR)	4
No. 77	4
No. 283	3

The above-mentioned strains having NRRL-numbers have the same taxonomic characteristics as strain K-12, which in turn has the taxonomic characteristics given in "Bergey's Manual of Determinative Bacteriology" 8th Edition, and they were deposited at the Agricultural Research Culture collection (NRRL) (an International Depositary Authority under the Budapest Treaty) on the 11 March 1981.

Claims

1. An L-methionine-producing microorganism obtained by incorporating, into a recipient strain of the genus *Escherichia,* a hybrid plasmid having had inserted therein a DNA fragment possessing genetic information relating to L-methionine production, which fragment is derived from a donor strain of the genus *Escherichia* resistant to ethionine.
2. A microorganism as claimed in claim1, wherein said recipient strain is of *Escherichia coli.*
3. A microorganism as claimed in claim 2, wherein said recipient strain is *Escherichia coli* K-12 or a mutant thereof.
4. A microorganism as claimed in any of claims 1 to 3, wherein said donor strain is of *Escherichia coli.*
5. A microorganism as claimed in claim 4, wherein said donor strain is *Escherichia coli* K-12 or a mutant thereof.
6. A microorganism as claimed in any of claims 1 to 5, wherein said hybrid plasmid is derived from pBR 322.
7. *Escherichia coli* AJ 11539 (NRRL B-12399).
8. *Escherichia coli* AJ 11540 (NRRL B-12400).
9. *Escherichia coli* AJ 11541 (NRRL B-12401).
10. *Escherichia coli* AJ 11542 (NRRL B-12402).
11. A process for producing L-methionine, which comprises culturing an L-methionine-producing microorganism as claimed in any of claims 1 to 10.
12. A process according to claim 11, substantially as described herein.
13. L-methionine produced by a process according to claim 11 or 12.

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